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# Selective targeting of cancer cells through inhibition of Checkpoint kinase 1

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Oslo, September 2012

Grete Hasvold

## List of papers

List of papers included in the thesis, referred to as papers I-III in the text:

**Paper I**

Petersen L*, Hasvold G*, Lukas J, Bartek J, Syljuåsen RG.

*p53-dependent G1 arrest in 1st or 2nd cell cycle may protect human cancer cells from cell death after treatment with ionizing radiation and Chk1 inhibitors.*

Cell Prolif. 2010 Aug; 43(4):365-71.*Shared first authorship.

**Paper II**

Hasvold G, Nähse-Kumpf V, Tkacz-Stachowska K, Rofstad EK, Syljuåsen RG.

*The efficacy of CHK1-inhibitors is not altered by hypoxia, but is enhanced after reoxygenation.*

Submitted.

**Paper III**

Hasvold G*, Lund-Andersen C*, Lando M, Lyng H, Suo Z, Syljuåsen RG.

*Impact of hypoxia on G2 checkpoint signaling.*

Manuscript. *Shared first authorship.

Other papers by the author not included in the thesis:

Landsverk HB, Mora-Bermúdez F, Landsverk OJ, Hasvold G, Naderi S, Bakke O, Ellenberg J, Collas P, Syljuåsen RG, Küntzinger T.

*The protein phosphatase 1 regulator PNUTS is a new component of the DNA damage response.*

EMBO Rep. 2010 Nov;11(11):865-75.

Halle C, Andersen E, Lando M, Aarnes EK, Hasvold G, Holden M, Syljuåsen RG, Sundfør K, Kristensen GB, Holm R, Malinen E, Lyng H.

*Hypoxia-induced gene expression in chemoradioresistant cervical cancer revealed by dynamic contrast enhanced MRI.*

Cancer Res. 2012 Aug 13. Epub ahead of print

# Introduction

Cells are constantly exposed to various stresses that damage their DNA, either through endogenous sources such as reactive oxygen species (ROS) that form as byproducts of cellular metabolism, or exogenous sources such as UV light from the sun, radioactive isotopes, or various chemicals in the environment. This damage has to be repaired efficiently and correctly in order to maintain cell viability and avoid genomic instability and malignant transformation. To overcome the massive task of maintaining the integrity of the genome in such a hostile environment, cells have developed a series of mechanisms for DNA damage repair, delay of cell cycle progression, and induction of controlled cell death should repair fail, collectively termed the DNA damage response (DDR)[1-3]. In the process towards malignant transformation, cancer cells often lose the function of one or more regulators of the DDR, and mutations in genes encoding DDR proteins are found in several cancer predisposition syndromes. In addition, the microenvironment found in solid tumors often differs considerably from that of normal tissues, and these differences may also have a profound effect on the regulation of the DDR in cancer cells.

While failure to repair damaged DNA may lead to cancer, DNA damage is also used to cure cancer, and in this regard the DDR insufficiencies of cancer cells may be exploited to improve the therapeutic ratio of cancer treatments. However, cancer is not a homogenous disease, and finding what subgroups of tumors will respond best to certain treatments requires extensive knowledge of the molecular mechanisms involved in mediating treatment efficacy.

The potential for targeting cancer specific traits by inhibition of the DDR protein Checkpoint kinase 1 (Chk1) has been the main focus of this thesis. This introduction aims to give an overview of the DDR in response to ionizing radiation (IR), the role of Chk1 in both the DDR and normal cell cycle progression, as well as the role of tumor hypoxia in modulating the DDR and the efficacy of Chk1 inhibition.

## The ionizing radiation-induced DNA damage response

### DNA damage induced by IR

Current cancer treatments include three main groups of therapy: Surgery, chemotherapy and radiation therapy. Most patients are treated with a combination of these treatments, depending on the type of cancer, localization and size of the primary tumor, metastatic spread and other factors. As the name implies, IR is photons or particles that have the ability to eject electrons from molecules. The resulting ionizations may either occur directly in critical targets or generate free

radicals which can interact with other molecules such as water or molecular oxygen, creating more stable radicals that can diffuse far enough to reach and ionize critical targets [4]. In cells, the majority of the biological damage from X-ray or γ-ray radiation is caused by such secondary free radicals [5]. While all molecules in the cell may become damaged in a randomly distributed manner, most of these molecules, such as proteins and mRNA, are present in multiple copies and have a rapid turnover, thus limiting the consequence of IR-induced damage to them. In contrast, DNA is only present in two copies, has a very low turnover if any, is vital for all cellular functions and constitutes the largest target [4, 6]. IR causes several types of damage to the DNA, including single-strand breaks (SSBs), damaged bases, double-strand breaks (DSBs) and DNA-DNA and DNA-protein crosslinks [5, 7]. A dose of 1Gy is estimated to result in ~$10^5$ ionizations, >$10^3$ damaged bases, approximately $10^3$ SSBs and 20-40 DSBs [5], but will only result in death of about 30 percent of the cell population for a typical mammalian cell line due to the activity of efficient repair pathways. Of these insults, DSBs are considered the most lethal[8], even though they are far outnumbered by the other forms of damage. DSBs are repaired through two main mechanisms: Non-homologous end-joining (NHEJ) and homologous recombination repair (HR). These mechanisms will be further described in a later section. SSBs and damaged based are repaired via the closely related SSB repair and base-excision repair pathways, respectively, while crosslink repair is dependent on the Fanconi anemia (FA) pathway. Cells also have specialized mechanisms for repair of mismatched bases (mismatch repair (MMR)) and bulky lesions (nucleotide excision repair (NER)), which typically occur during replication and in response to UV-light, respectively. However, these repair mechanisms are less relevant for IR-induced damage[9].

### DNA damage sensors

The first step in activation of the DDR following IR-induced DNA damage is the sensing of the damage and recruitment of proteins that control repair, checkpoint and cell death pathways. Three kinases of the PIKK (phospho-inositide3-kinase related kinases) family play fundamental roles in this process[1]. ATM (Ataxia telangiectasia mutated) is the main kinase in response to DSBs, while DNA-PK (DNA-dependent protein kinase) responds to sensors involved in the NHEJ pathway. ATR (ATM-and Rad3 related) is recruited to sites with stretches of single-stranded DNA (ssDNA), which includes stalled replication forks and resected SSBs and DSBs. All three of these kinases are able to phosphorylate the C-terminal Ser139 on the histone variant H2AX (known as γH2AX)[10], one of the first steps in forming IR-induced foci (IRIF), microscopically visible regions of accumulated DDR proteins [11].

### *MRN and ATM*

DSBs are initially recognized by the MRN complex, which includes the Mre11 nuclease, DNA binding protein Rad50 and ATM-interacting Nbs1 (Nijmegen breakage syndrome 1) (reviewed in [12-14]). Binding of ATM to Nbs1 activates ATM [15], which then phosphorylates itself [16, 17], MRN and several other downstream targets including H2AX. This creates a docking site for the recruitment of the mediator protein MDC1 (mediator of DNA damage checkpoint protein 1) which binds γH2AX via its BRCT-domain [18]. MDC1 also binds ATM, leading to further recruitment of ATM to the damage site, initiating a positive feedback loop of protein accumulation and amplification of the DNA damage signal [19]. MDC1 is essential for the recruitment of the repair proteins 53BP1 and BRCA1 to the sites of damage via its interaction with the E3 ligase RNF8 (reviewed in [11]). MDC1 is also required for retention of Nbs1 at sites of DNA breaks [20, 21], which is important for maintaining the DDR signaling until repair is complete. Activation of ATM is also dependent on acetylation of Lys3016 by the acetyl-transferase Tip60[22, 23], which is recruited to DSBs by direct interaction with the MRN complex [24].

### *Ku and DNA-PKcs*

The Ku complex, composed of the subunits Ku70 and Ku80, competes with the MRN complex for binding to DNA ends and functions as a DSB sensor (reviewed in[25]). Ku recruits DNA-PKcs to the DSB, forming the active kinase complex referred to as DNA-PK, and this constitutes the first step in the process of NHEJ (reviewed in [26]). While Ku and DNA-PKcs are fundamental for this repair pathway, they do not play a major role in IRIF formation and phosphorylation of H2AX[25].

### *ATRIP and ATR*

ATR is not recruited directly by MRN to DSBs, but binds stretches of RPA-coated ssDNA that accumulate at resected breaks via its interaction partner ATRIP (ATR-interacting protein)[27]. MRN and ATM are required for the initiation of DSB resection, mediated by Mre11 and CtIP[28], but ATR eventually replaces ATM at the resected break as ATM loses its affinity for the resected DNA strand, resulting in a switch from ATM to ATR mediated signaling[29]. Activation of ATR is additionally dependent on the recruitment of the Rad9-Hus1-Rad1 (9-1-1) complex and TopBP1[30, 31]. The 9-1-1 complex is a trimeric ring-shaped clamp protein that resembles the PCNA (proliferating cell nuclear antigen) clamp involved in replication, and encircles the DNA at the damage site (reviewed in [32]). Loading of 9-1-1 onto DNA is facilitated by Rad17 in cooperation with four subunits of the replication factor C protein (RFC)[33]. TopBP1 binds both Rad9 and ATR-ATRIP, elevating the kinase activity of ATR[34, 35]. At stalled replication forks TOPBP1 can also interact with MDC1 [36], which may further regulate ATR activity. In addition, it was recently identified that RHINO (Rad9, Rad1 and Hus1 interacting nuclear orphan) binds both 9-1-1 and

TopBP1 at sites of DNA damage and is important for activation of ATR-mediated HR and checkpoint signaling, but the exact mechanism is not known [37].

## Cell cycle checkpoints

The two main DNA damage induced cell cycle checkpoints in mammalian cells are the G1/S and G2/M checkpoints, which halt progression of the cell cycle before entry into S phase and mitosis, respectively. In addition, mammalian cells exposed to DNA damage also show a delay in S phase progression, known as the S phase checkpoint. The main regulators of these DNA damage checkpoints are the ATR and ATM kinases in cooperation with the checkpoint kinases Chk1 and Chk2. As these kinases arrest cell cycle progression primarily by activating pathways that target the activity of cyclin-dependent kinases (CDKs) (**Figure 1**), I will first briefly review the function of CDKs in normal cell cycle progression.

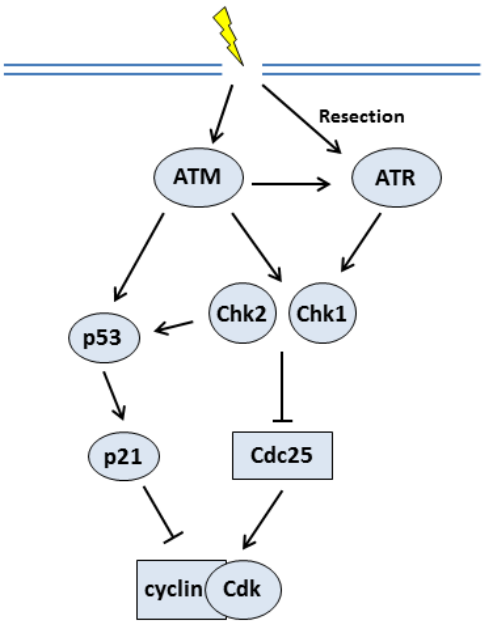


**Figure 1. Activation of DNA damage-induced cell cycle checkpoints.** In response to DSBs, ATM is activated. Active ATM phosphorylates the checkpoint kinases Chk1 and Chk2, leading to inhibition of the Cdc25 phosphatases. Cdc25 will then no longer be able to remove the inhibitory phosphorylation on the Cdk-cyclin complexes, and CDK activity decreases. ATM and Chk2 also promote activation of p53, leading to transcriptional upregulation of the CDK inhibitor p21. Checkpoint activation is also promoted by ATR in response to resected DSBs, leading to activation of Chk1 and decreased Cdc25 activity. Decreased CDK activity leads to cell cycle arrest by inhibiting replication initiation and mitotic entry. See main text for details and references.

### *Basic regulation of cell cycle progression by CDKs*

The CDKs are a family of related Ser/Thr kinases, and as the name implies, their activity is dependent on their interaction with cyclins, whose expression is restricted to distinct phases of the cell cycle (see[38] for a comprehensive overview of cell cycle regulation by CDKs) (**Figure 2**). In early G1 Cdk4 and Cdk6 are the active CDKs forming complexes with the D-type cyclins (D1, D2 or D3).

The Cdk4/6-cyclin D complexes phosphorylate the Retinoblastoma protein (pRb), abrogating its inhibitory interaction with the transcription factor E2F. Active E2F induces the expression of several S phase proteins, including cyclin E which binds to Cdk2, initiating a positive feedback loop of increasing CDK activity, phosphorylation of pRb and E2F activity. The inactivation of pRb occurs several hours before cells enter S-phase, but marks the restriction point at which the cells are committed to start a new cell cycle (reviewed in [39]).

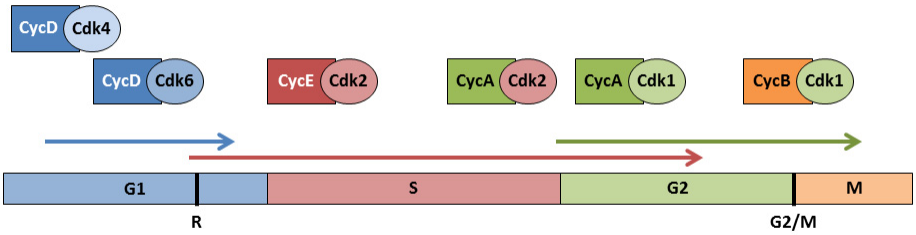


**Figure 2. Overview of CDK complexes promoting cell cycle progression.** R marks the restriction point where cells are committed to initiate a new round of cell division. G2/M marks the transition from interphase to mitosis, another point of no return.

As cells progress from G1 into S phase, cyclin E is degraded and Cdk2 interacts more with cyclin A, which later in S phase also interacts with Cdk1 (Cdc2). In G2, the level of B-type cyclins (mainly B1) increase and the Cdk1-cyclin B1 complex promotes mitotic entry. In addition, the Cdk1-cyclin A complex can also contribute to drive G2 cells into mitosis [40]. In mitosis, the S and M phase cyclins are degraded by the anaphase-promoting complex or cyclosome (APC/C), thus efficiently turning off the activity of the CDKs. The APC/C is a multisubunit ubiquitin-protein ligase, and its activity is dependent on activator subunits, Cdc20 in early mitosis and Cdh1 in late mitosis and early G1 (reviewed in [41]).

In addition to cyclin binding, CDK activity is regulated by binding of CDK inhibitors such as p16, p21 and p27 and phosphorylation and dephosphorylation events. Phosphorylations that promote CDK activity are carried out by the CDK-activating kinase (CAK) [42] and include Thr161 in Cdk1 and Thr160 in Cdk2. Phosphorylations that inhibit Cdk1 and Cdk2 activities include Tyr15 and to some extent Thr14 residues and are carried out by the Wee1 and Myt1 kinases. These inhibitory phosphorylations are removed by the activity of the Cdc25 phosphatases (Cdc25A, Cdc25B and Cdc25C)[43]. Cdc25A was first thought to primarily activate the Cdk2-cyclin E and Cdk2-cyclin A complexes at the G1/S transition[44], but later studies have shown that it also activates the Cdk1-cyclin B1 complex at the G2/M transition[45, 46]. Cdc25B and Cdc25C mainly regulate the activity

of the Cdk1-cyclin B1 complex in G2 and M [47], though they have also been implicated in the regulation of S phase entry [48, 49]. Even so, Cdc25B and Cdc25C have been shown to be dispensable for normal cell cycle progression and checkpoint responses in mice [50]. Knockout of Cdc25A results in embryonic lethality, while Cdc25B and Cdc25C may compensate for lack of Cdc25A in adult tissues [51]. These results indicate that there is considerable redundancy between the family members.

### *G1 checkpoint*

Activation of the G1 checkpoint is mainly mediated by the ATM-Chk2-Cdc25A and ATM-p53-p21 pathways (reviewed in [52]), and is reported to be independent of ATR and Chk1 [53], though Chk1-mediated signaling for the targeting of Cdc25A may be involved in late G1 [54]. The response targeting Cdc25A is a fast and transient process, while the p53-p21 mediated pathway is a slow and potentially permanent arrest that involves transcriptional upregulation of the CDK inhibitor p21. Activated ATM at sites of DNA damage recruits and activates Chk2, mainly by phosphorylation of the Thr68 residue of Chk2 [55]. Activated Chk2 phosphorylates Cdc25A[56], marking this phosphatase for degradation, and with Cdc25A no longer available to remove the inhibitory Tyr15 and Thr14 phosphorylations on Cdk2, CDK activity is decreased. However, this only results in a reduced rate of S phase entry within the first 4-6 hours after genomic insult (such as IR-treatment), while a full arrest can be observed in G1 phase at later time-points [52]. This later arrest is dependent on p53 and p21, and is often lacking in cancer cell lines. ATM and Chk2 both phosphorylate p53 [57-59] and its negative regulator Mdm2 [60, 61], resulting in accumulation and activation of p53. Active p53 transcriptionally upregulates p21, which binds to and inactivates Cdk2-cyclin E and Cdk4/6-cyclin D complexes [62], thus preventing progression into S phase. Inhibition of the CDK complexes in early G1 prevents initiation of the positive feedback loop of pRb phosphorylation and release of the E2F transcription factors, keeping the cells from passing the restriction point and enabling the option of permanent G1 arrest [52].

### *S phase checkpoint*

As is the case in late G1, activation of the intra-S phase checkpoint after IR can only delay cell cycle progression, but not induce permanent arrest. This delay in S phase progression is mediated by the same mechanisms as the immediate G1 checkpoint, the inhibition of CDK activity by targeting of Cdc25A for degradation via phosphorylation by Chk1 and Chk2 downstream of ATM and ATR [63]. IR-induced decreased CDK activity prevents the firing of new replication origins [64, 65] and slows down replication fork progression [66](see also section on Chk1 and regulation of CDK activity), thus delaying the rate of replication and extending the time spent in S phase. In addition to the initial IR-induced DSBs, the progression of the replication forks can induce secondary DNA damage

that may contribute to the activation of ATR and Chk1 mediated signaling and repair pathways [67].

### *G2 checkpoint*

Cells irradiated in G2 activate an immediate and transient arrest before mitotic entry by targeting the interaction between Cdk1 and the mitotic cyclin B1 via the ATM-Chk1-Cdc25 pathway, similar to what is observed in the other cell cycle phases. Chk2 does not appear to play a role in G2 checkpoint regulation, though studies on this subject have been conflicting (reviewed in [68, 69]). Cells that were irradiated in S phase will also accumulate in late G2 until repair is completed, as the S phase checkpoint can only delay, but not arrest cell cycle progression, as described above. However, this late arrest is independent of ATM, depending instead on ATR-Chk1 signaling and may last for many hours after higher doses of IR [70]. The dependence of the G2 accumulation on ATR-Chk1 may reflect the type of damage that remains at these later time-points [67]. Repair by NHEJ is a rather quick process, and breaks that are not efficiently repaired by this mechanism are further resected to promote HR, which is a slow process that may take several hours to complete [71]. Indeed, several factors involved in HR have also been shown to be important for activation and/or maintenance of the late G2 checkpoint, including BRCA1, BRCA2 and its interaction partner PALB2 [37, 72, 73]. This repair pathway is described in the next section. The role of p53 and p21 in G2 arrest has been unclear in previous studies (reviewed in [52]), but a recent study found that both Chk1 and p21 are required for sustained G2 arrest in cancer cell lines while Chk1 may be dispensable when the ATM/p53/p21 pathway is fully functional [74]. Mitotic entry after induction of G2 checkpoint arrest requires the cessation of checkpoint signaling and the reactivation of the Cdk1-cyclin B1 complex (reviewed in [75]). This process is dependent on the kinase Plk1 (Polo-like kinase 1), which targets both Wee1 and the Chk1 regulator Claspin (described below) for proteasomal degradation [76-79]. Activation of Plk1 is dependent on the Bora-Aurora A kinase complex [80], which also regulates unperturbed mitotic progression [81]. In addition to Plk1, checkpoint recovery is also dependent on the activity of several phosphatases that remove the activating phosphorylations on Chk1 and other checkpoint proteins (reviewed in [82]). These phosphatases include PP2A (protein phosphatase 2A)[83] and Wip1 (wild-type p53-induced phosphatase 1)[84, 85].

## DNA double-strand break repair

As previously mentioned, mammalian cells have two main pathways for repair of DSBs: The “quick and dirty” option of NHEJ and the slow but error-free HR (**Figure 3**). While NHEJ can take place in all phases of the cell cycle, HR is mainly restricted to S and G2 phases, where a sister chromatid is

available. Both repair pathways are initiated by binding of the MRN complex and activation of ATM, but different protein complexes are involved in the subsequent steps of repair.

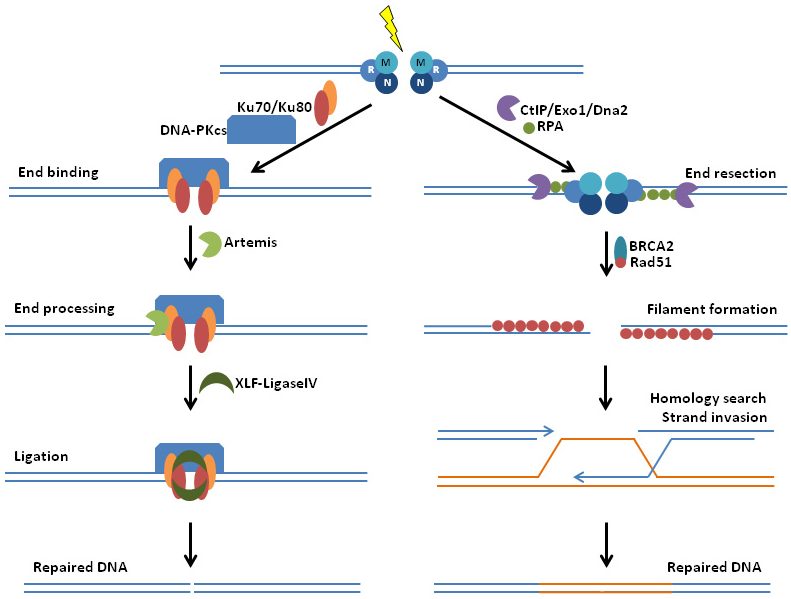


**Figure 3. Simplified overview of the NHEJ and HR repair pathways.** DSBs are initially recognized by the MRN complex. Replacement of the MRN complex by the Ku70/Ku80 dimer promotes recruitment of DNA-PKcs and repair by the NHEJ pathway. The nuclease Artemis initiates simple end processing, and a ligase complex including XLF and Ligase IV mediates end ligation of the processed ends, resulting in repair of the DSB with minimal resection taking place. In S and G2 phases, where CDK activity is high, end resection of the DSB is initiated by CtIP, followed by further resection mediated by Exo1/Dna2. RPA binds the resulting stretches of ssDNA. RPA is replaced by Rad51, whose loading is facilitated by BRCA2. Rad51 filaments in association with other HR factors then initiates homology search and strand invasion at the sister chromatid, using this as a template for new stretches of DNA, resulting in error-free repair of the DSB. See main text for references.

### *Homologous recombination repair*

HR is dependent on 5' to 3' resection of the DSB, which is initiated by the Mre11 nuclease in cooperation with CtIP [28]. The association of CtIP to the MRN complex is dependent on BRCA1 [86]. Further resection is carried out by the exonuclease Exo1 or nuclease Dna2 in cooperation with the helicase BLM [87], resulting in long stretches of ssDNA that are immediately coated by RPA. RPA is then replaced by the recombinase Rad51, whose recruitment to DSBs is dependent on BRCA2 and its interaction partner PALB2 (reviewed in [88]), creating a nucleoprotein filament that initiates homology search, strand invasion and DNA synthesis by an unknown polymerase using the

sister chromatid of the damaged stretch as a template [89], resulting in error-free repair of the damaged DNA.

### *Non-homologous end-joining*

NHEJ is initiated by the binding of the Ku complex to the break ends, which replaces the MRN complex and thus removes ATM from the break site (reviewed in [90]). As previously mentioned, the Ku complex is a heterodimer composed of the Ku70 and Ku80 proteins, and their binding to DSBs recruits DNA-PKcs to the DSB. Binding and activation of DNA-PKcs leads to recruitment and activation of the nuclease Artemis, in addition to the other necessary repair proteins, which include the μ and λ polymerases and a ligase complex (XLF with DNA ligase IV), resulting in an efficient ligation of the DNA strands with minimal resection taking place (reviewed in [26]).

### *Choice between HR and NHEJ*

The shift between NHEJ and HR mediated repair is primarily mediated by the initial resection taking place at the DSB, which also shifts the DDR response from ATM to ATR mediated signaling. The resection is regulated by CDKs, which phosphorylate CtIP in addition to several other factors involved in both initial DSB sensing and HR repair (reviewed in [91, 92]). CtIP activity is dependent on phosphorylation on Thr847 [93], while its interaction with BRCA1 and thus recruitment to sites of damage depends on phosphorylation on Ser327 [86]. BRCA1 is also regulated by CDKs, being phosphorylated on Ser1497 by Cdk2 [94] and at Ser1497 and Ser1189/Ser1191 by Cdk1 [95]. These phosphorylations are likely to be important for Rad51 recruitment [91]. CDKs have also been reported to phosphorylate Mre11 [96], Nbs1 [97] and BRCA2 [98], regulating their activity and/or interactions with other proteins. Confinement of HR to S and G2 phases thus appears to be largely regulated by CDK activity, and is yet another example of the extensive crosstalk taking place between the DDR and cell cycle machinery.

While CDK activity and regulation of resection may restrict the use of HR to S and G2, NHEJ is used throughout the cell cycle and is the major pathway for repair of direct two-ended DSBs also in S and G2 phase [99]. HR appears to be favored in the repair of more complex and persisting two-ended DSB (reviewed in [100]), in particular for DSBs in heterochromatic regions [101, 102], and is also required for repair of one-ended DSBs formed by collapsed replication forks [103, 104]. Interestingly, a recent study showed that loss of NHEJ proteins increases resection, suggesting that ongoing NHEJ suppresses HR repair [71].

## Cell death after IR

When cells are unable to sufficiently repair the damage induced by IR, they can lose their ability to proliferate or undergo cell death through various pathways. How, when and why cells die after IR varies between different cell types, with some being more prone to apoptosis while others hardly ever die by this mechanism[105]. The timing and mechanism of IR-induced cell death may even be different for individual cells within a homogenous population. For cells that undergo a few cell divisions after being irradiated, each of the daughter cells may die in different ways and at different times[106, 107]. Nevertheless, the susceptibility to certain modes of cell death can be an important factor in determining the radiation sensitivity of tissues. Cancer cells often display mutations in these pathways, and may therefore respond differently to IR than the normal tissue from which they originated.

### *Apoptosis*

Apoptosis is a tightly controlled mode of cell death that is an essential part of embryonic development, tissue homeostasis, immune system regulation and several other normal physiological processes (reviewed in [108-110]). It may be activated via an extrinsic pathway regulated by the binding of extracellular ligands to death receptors or an intrinsic pathway dependent on intracellular signaling, such as in the response to DNA damage. Both pathways are initiated by caspases, a family of cysteine proteases which under normal conditions are kept in an inactive form known as procaspases[111]. The intrinsic pathway is initiated by caspase 9, whose activation is primarily regulated by the balance of pro- and anti-apoptotic proteins [112]. These proteins include the anti-apoptotic BCL2 and pro-apoptotic BAX and PUMA, the latter two being induced by p53 activation. These proteins reside in or near the mitochondria and a shift in favor of pro-apoptotic proteins results in the release of cytochrome c and other mitochondrial proteins into the cytoplasm, triggering the formation of a structure known as the apoptosome and subsequent activation of caspase 9. Caspase 9 initiates a signaling cascade of caspase activation, resulting in the cleavage of a large set of cellular proteins by effector caspases (including caspase 3) leading to cell death. Cells that die via apoptosis display a distinct morphology that includes membrane blebbing, DNA fragmentation, chromatin condensation and packaging of cellular components into membrane-enclosed apoptotic bodies that in vivo are taken up and digested by phagocytes. This highly ordered mode of cell death and breakdown of components prevents inflammation and damage to the surrounding tissue, unlike what is observed when cells burst and die via necrosis.

### *Autophagy*

Autophagy literally means “self-eating”, and is primarily a mechanism for survival in stressful conditions such as growth factor deprivation, starvation or hypoxia, though extensive autophagy

does lead to cell death [113]. This involves the digestion of mitochondria and other larger cellular structures. Autophagy is characterized by some of the same morphological changes observed in apoptotic cells, including membrane blebbing and chromatin condensation, but no DNA fragmentation.

### *Necrosis*

Necrosis is characterized by cell swelling, breakdown of the extracellular membrane, non-condensed chromatin and generally a disorganized cellular structure. This is considered to be an "accidental" and uncontrolled form of cell death, which typically occurs in response to extreme conditions of extracellular pH changes, ion imbalance or energy loss, but also in response to IR-induced DNA damage. Necrosis is also phenotypically similar to the regulated process of necroptosis, and may itself be a more regulated process than initially assumed (reviewed in [114]).

### *Senescence*

When cells permanently lose their ability to divide, either due to shortening of telomeres or in response to irreparable DNA damage, they enter a state of cell cycle arrest termed senescence (reviewed in [115]). These cells display a distinct morphological phenotype that includes a flattened cytoplasm, high granularity and often enlarged size. They also have increased expression of β-galactosidase, which is commonly used as a biochemical marker to detect senescent cells, and may have increased regions of heterochromatin. Induction of senescence in response to DNA damage appears to be mainly regulated by the p53/p21 and pRb/p16 pathways.

### *Mitotic catastrophe (mitosis-linked death)*

Cancer cells often have mutations in genes involved in the regulation of apoptosis and senescence (such as p53, which is involved in both), and may survive massive amounts of unrepaired DNA damage provided they do not attempt cell division. Mitotic catastrophe, or mitosis-linked death, is a term that refers to cell death that occurs as a result of or following aberrant mitosis, and is frequently observed in irradiated cells (reviewed in [116]). Cells that undergo mitotic catastrophe typically display multiple nuclei, uncondensed chromatin, micronuclei/nuclear fragments and chromosomal aberrations. These cells may later die via any of the other cell death mechanisms (apoptosis, necrosis, senescence or autophagy), but this death occurs as a consequence of cells undergoing mitosis in the presence of unrepaired or misrepaired DNA damage, and is not directly triggered by the initial damage.

## Checkpoint kinase 1

While Chk1 is a key regulator of the IR-induced S and G2 checkpoints, as described above, it also plays a role in normal cell cycle progression. Chk1 null mice die early in embryogenesis, while mice with heterozygous knockout of Chk1 are reported to be overtly normal [117-119]. However, conditional knockout of one allele of Chk1 in adult mouse mammary epithelial tissue resulted in activation of an extensive DDR, showing Chk1 to be haploinsufficient for normal growth in such tissues [120]. Abrogation of Chk1 function by inhibitors or knockdown by interfering RNA (shRNA or siRNA) in mammalian cell cultures results in increased replication initiation followed by replication stalling and DNA breakage [121], in addition to premature mitotic entry [122] and aberrant mitosis [123]. These results show that Chk1 is not only required for checkpoint responses following DNA damage, but also for maintenance of genomic integrity via multiple functions in the unperturbed cell cycle. This chapter will present some of the functions of Chk1 in these vital processes, both in unperturbed and damaged cells, and how these functions may be targeted to improve cancer therapy.

### Activation of Chk1

As previously mentioned in the chapter regarding DNA damage induced cell cycle checkpoints, Chk1 is activated by ATR (and to some extent ATM) at sites of RPA-coated ssDNA (reviewed in [124] and [125]). ATR phosphorylates Chk1 on Ser317 and Ser345 [117, 126-128], which is assumed to alleviate inhibitory intramolecular interactions between the N-terminal kinase domain and regulatory C-terminal domain of Chk1, allowing Chk1 to assume an active conformation and phosphorylate itself at Ser296 [129]. However, it is unclear whether the C-terminal domain of Chk1 actually binds the N-terminal domain [124, 128, 130, 131], and Chk1 has been shown to be in an open conformation also in its inactive and non-phosphorylated state [130]. Recently, an alternative splice variant of Chk1, Chk1-S, was shown to function as an endogenous inhibitor of Chk1 [132], consistent with the model of a repression factor binding the C-terminal domain to regulate Chk1 activity rather than this domain folding back on the N-terminal kinase domain [128]. Following phosphorylation of Ser296, the Ser317 and Ser345 residues are dephoshorylated by phosphatases including PP2A [133, 134] and Wip1 [85], facilitating release of Chk1 from chromatin and spreading to the nucleoplasm, where it can interact with its targets such as Cdc25A. Phosphorylated Ser296 on Chk1 acts as a binding motif for 14-3-3γ, which promotes nuclear retention of Chk1 and mediates the interaction between Chk1 and Cdc25A [135]. The C-terminal regulatory domain of Chk1 is also phosphorylated by other kinases than ATR. Ser286 and Ser301 are phosphorylated by CDKs in mitosis and in response to replication stress [136, 137], while Ser280 is phosphorylated by p90 ribosomal S6 kinase (p90 RSK) in response to growth factor stimulation [138].

### Chk1-mediated regulation of CDK activity

While the activity of ATR and Chk1 increases in response to DNA damage, these proteins are also active in the normal unperturbed cell cycle, regulating the levels and activity of the Cdc25 phosphatases, and thus the CDK activity. Most of the detrimental effects observed in response to Chk1-inhibition are mediated by deregulated CDK activity [139], and may be alleviated by co-depletion of Cdc25A [140]. As mentioned in the section on cell cycle checkpoints, Chk1 phosphorylates Cdc25A, Cdc25B and Cdc25C, preventing these phosphatases from removing the inhibitory phosphorylations on Tyr15 and Thr14 on Cdk1 and Cdk2. Chk1-mediated phosphorylation of Cdc25A leads to inactivation of Cdc25A via degradation[63, 135]. Chk1 phosphorylates Cdc25A on Ser76, and also activates the kinase Nek11 (Never in mitosis gene-A related kinase 11) which phosphorylates Cdc25A on Ser82, marking it for β-TrCP-SCF dependent ubiquitination and proteosomal degradation [141]. In the absence of Chk1, Cdc25A accumulates [63], resulting in increased activity of CDK complexes, with subsequent detrimental effects on replication and mitosis as discussed below. In contrast to Cdc25A, Cdc25B and Cdc25C are not degraded in response to Chk1-mediated phosphorylation, but their inactivation is also dependent on interaction with 14-3-3 proteins. Phosphorylated Ser323 on Cdc25B and Ser216 on Cdc25C [142, 143] form binding motifs recognized by 14-3-3 proteins, which sequester Cdc25C in the cytoplasm and prevent Cdc25B from interacting with the Cdk1-cyclin B complex [144]. Chk1 may also regulate CDK activity by phosphorylating and activating Wee1 [139, 145], the kinase that puts on the inhibitory Tyr15 phosphorylation on Cdk1 and Cdk2.

### Chk1 and DNA replication

Replication of mammalian genomes is initiated at multiple sites called origins of replication, marked by the binding of proteins forming the origin recognition complex (ORC) (reviewed in [146]). In late M and early G1 phase ORCs recruit Cdc6 and Cdt1, which are responsible for ATP-dependent loading of the MCM helicase, a structure composed of MCM2-7 proteins that encircle the DNA double-strand and is loaded as bi-directional double hexamers, forming the pre-replication complex (pre-RC). The MCM complexes are initially inactive as helicases until early S-phase, when the pre-RCs are phosphorylated by CDK (Cdk2) and Cdc7-Dbf4 (DDK) kinases, triggering recruitment of Cdc45 and And1-Ctf4 via Treslin and GEMC1 [147, 148]. And1 in association with its interaction partners Tim1 and Tipin is crucial for the recruitment of polymerase α and replication initiation [149], while Cdc45 in cooperation with the GINS heterotetrameric complex stimulate the activity of the MCM helicase to form a productive replication fork [150].

Licensed origins are activated in a highly regulated manner and at different times during S phase within replication factories, discrete clusters of chromatin coupled to replication machinery

proteins [151]. In the course of a normal S phase, only a small fraction of the licensed origins are fired, with the majority of the origins being passively replicated by adjoining replication forks. However, in response to replication stress these dormant origins may be fired to compensate for lack of replication at stalled forks. Activation of replication factories may be regulated separately from origin activation within factories by CDKs [152, 153]. Activated Chk1 suppresses initiation of origin firing [154]. Such Chk1-mediated suppression of origins occur particularly in new replication clusters, while Chk1 can promote firing of dormant replication origins within ongoing replication clusters [155]. This allows for the continued replication of ongoing regions despite decreased total rate of DNA synthesis in response to Chk1 activation, as is observed after activation of the S phase checkpoint. Inhibition of Chk1 in normal S phase cells, and subsequent elevation of CDK activity due to accumulation of Cdc25A, leads to increased Cdc45 loading at replication origins, unscheduled initiation and accumulation of ssDNA near replication origins, followed by massive replication stalling and formation of DSBs [121](**Figure 4**). Thus, Chk1 is required during normal S phase progression to prevent DNA breakage and maintain genome integrity.

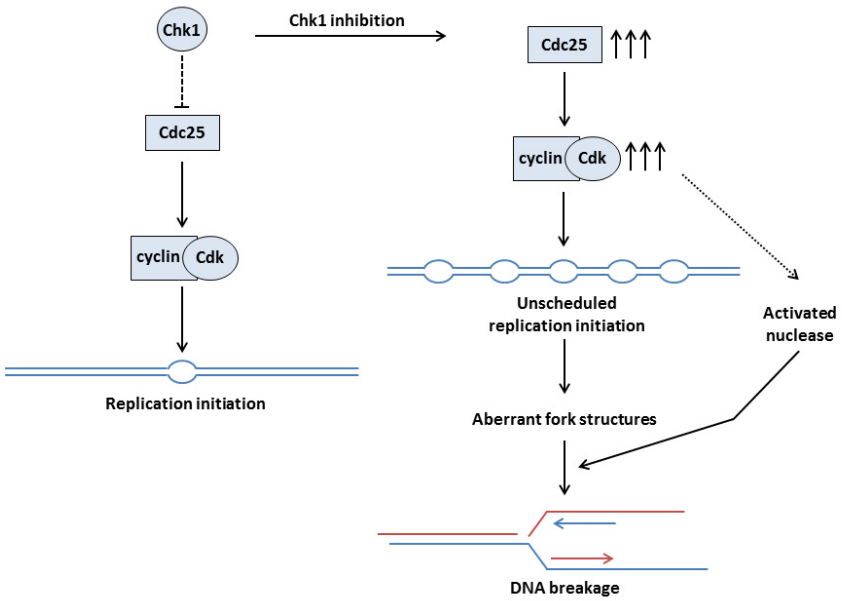


**Figure 4. Consequences of Chk1 inhibition in S phase cells.** In the unperturbed S phase, Chk1 regulates CDK activity through maintenance of Cdc25 levels. CDK activity regulates replication initiation, and in the absence of Chk1 Cdc25 accumulates, resulting in increased CDK activity. This leads to unscheduled replication initiation followed by replication stalling and formation of aberrant fork structures. Such structures are processed by nucleases, whose activity is also regulated by CDK activity, resulting in DNA breakage. Figure is adapted from [139].

While deregulated CDK activity may be the instigator of the unscheduled replication initiation in response to Chk1 inhibition, Chk1 has additional roles in replication and maintenance of genomic stability in S phase. Both ATR and Chk1 are directly bound to replication forks via RPA and Claspin in a complex with the replisome-associated proteins Tim and Tipin in addition to several other factors involved in replication [156], and are required for stabilization of stalled replication forks [157, 158]. Stalled replication forks often occur in regions that are difficult to replicate, such as common fragile sites [159], but may also be caused by such factors as lack of nucleotides or DNA bound protein complexes that hinder the progression of the polymerase [160]. Claspin is required for activation of Chk1 by ATR[161, 162], functioning as a bridge between these kinases, and is also required for recruitment and phosphorylation of BRCA1[163]. Phosphorylation of Claspin by Casein kinase 1 (CK1) promotes its interaction with Chk1 [164], which correspondingly promotes the stability of Claspin via a kinase-independent and ATR-independent mechanism [165]. When stalled replication forks collapse, they form a DSB that requires the HR pathway for repair. Chk1 directly regulates this repair pathway by phosphorylating Rad51 [166], a prerequisite for the activation of Rad51 and formation of the filaments that perform homology search and strand invasion. Chk1 also regulates the replication checkpoint, whose function is to ensure the completion of DNA replication before chromosomal condensation and mitotic entry as described below.

### Chk1 and mitosis

Mitotic entry and progression is orchestrated through several positive and negative feedback loops of kinases and phosphatases (reviewed in [167]). The main regulator of this process is the Cdk1-cyclin B complex, whose activity is dependent on the Cdc25 phosphatases as described above. Cdk1-cyclin B activity inhibits the Wee1 and Myt1 kinases, while activating Cdc25 family members, forming a positive feedback loop for increasing Cdk1 activity (reviewed in [47]). Chk1 is found at the centrosomes in interphase [122], where it is assumed to shield Cdk1 from interacting with Cdc25B. In the absence of Chk1, the Cdk1-cyclin B complex is prematurely activated, resulting in aberrant mitotic entry and disruption of the replication and spindle assembly checkpoints. Cells lacking Chk1 may thus enter mitosis before completion of replication [168], with the concomitant risk of chromatid breakage, genomic instability and/or death via mitotic catastrophe. In response to exogenous DNA damage, mitotic entry is also prevented via Chk1 mediated phosphorylation of Cdc25A[46]. Furthermore, Chk1 targets the activity of the mitotic regulators Plk1 [169] and Aurora-B [170], influencing both checkpoint recovery and cytokinesis. Indeed, the mitotic function of Chk1 linked to the phosphorylation of its Ser345 residue has been reported to be the essential function of this kinase, whereas the Ser317-dependent replication and DDR functions are non-essential [171].

### Rationale for using Chk1 inhibitors in cancer treatment

While Chk1 is involved in several aspects of both the DDR and normal cell cycle progression, it was initially its fundamental role in the regulation of the DNA damage induced S and G2 checkpoints that was the basis for the development of Chk1 inhibitors and their use in clinical trials to selectively target cancer cells [172-174]. Most cancer cells lack a functional G1 checkpoint, primarily due to mutation or loss of p53 or one or more factors involved in regulation of the p53 response, though other cell cycle regulators, such as pRb or CDKs, may be to blame. In contrast, mutations in Chk1 or ATR are rarely found in human cancers, although Chk1 frameshift mutations have been observed in some endometrial, melanoma, colon and stomach cancer with microsatellite instability [175-178]. Cancer cells with mutated p53 thus have only one functional DNA damage induced cell cycle checkpoint to depend on for survival following exposure to DNA damaging agents, and would therefore be expected to be selectively sensitized to DNA damage by

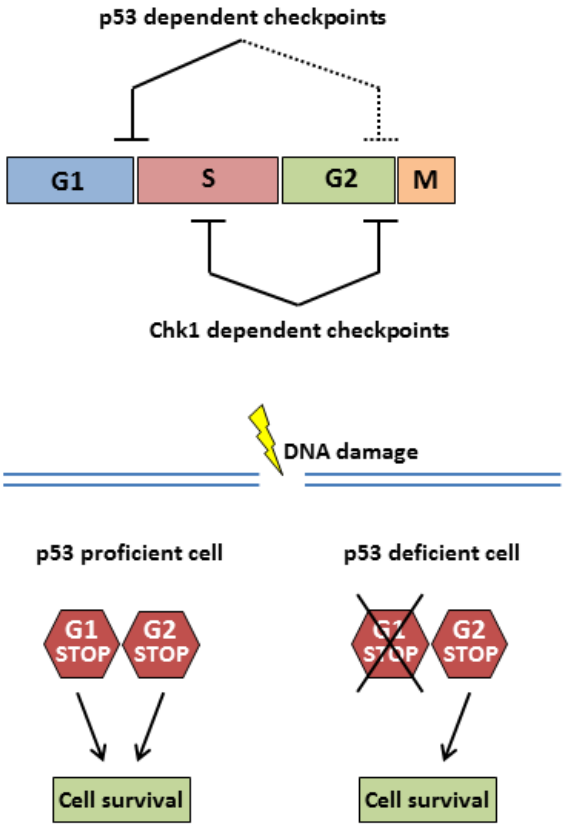


**Figure 5. Rationale for using inhibitors of Chk1 to target p53 deficient cells.** In response to DNA damage, cells depend on the activity of the G1, S and G2 checkpoints for survival. Chk1 regulates the S and G2 checkpoints, whereas the G1 checkpoint (and to some extent G2 checkpoint) is dependent on p53. Cancer cells often lack functional p53, and will therefore only have the S and G2 checkpoints to depend on for survival in response to DNA damage. These cells would therefore be expected to be selectively sensitized by inhibitors of Chk1, as they then have no functional checkpoints, whereas cells with functional p53 will still be able to arrest in G1.

abrogation of Chk1 and subsequent loss of the G2 checkpoint (**Figure 5**)[179, 180]. Though inhibition of Chk1 has been shown to enhance the effect of several DNA damaging agents such as IR, gemcitabine, anti-metabolites, cisplatin, topoisomerase I poisons and cisplatin (reviewed in [181]), the results regarding the importance of p53-status have been more inconclusive, which led us to initiate the study presented in paper I. The role of Chk1 in HR has also been suggested to be the main instigator of increased sensitivity to these DNA damaging agent rather than its role in G2 checkpoint regulation [182], though this may also be a means of selectively targeting rapidly dividing cancer cells. Rapidly growing cells will depend more on HR and less on NHEJ than non-cycling normal cells due to the cell cycle phase dependency of these repair pathways.

Of the DNA damaging agents tried out in combination with Chk1 inhibitors, the greatest sensitizing effects were observed with agents that induce a replication stress response, such as anti-metabolites and topoisomerase I poisons [180]. While Chk1 is rarely mutated in human cancer, it is frequently upregulated and/or activated in cancers with expression of replication stress-inducing oncogenes [183], such as Myc and Ras [184, 185]. Chk1 inhibitors have therefore been suggested as a means of selectively targeting cancers with high levels of oncogene-induced stress, such as melanomas [186] and Myc-driven lymphomas [187]. Activation of an ATR and Chk1-mediated replication stress response has also been observed in cells exposed to extreme hypoxia [188], and Chk1 inhibitors have therefore been proposed for selective targeting of such cells (see section on Hypoxia and activation of the DDR).

## Hypoxia

### Angiogenesis and tumor hypoxia

As a solid tumor forms, it outgrows it nutrient and oxygen supply, and must induce the formation of new blood vessels in order to grow further. This transition from a microscopic tumor to a macroscopic tumor with its own vascular system is known as the angiogenic switch [189]. Angiogenesis, or the formation of new blood vessels, is usually a tightly regulated process that is only turned on during distinct physiological processes such as embryogenesis or wound healing, and blood vessels in normal tissues have a well-organized and hierarchical structure to maintain an even distribution of nutrients and oxygen from the blood to the tissue. In contrast, the angiogenesis in solid tumors is a consequence of irregularities in the control of blood vessel formation, resulting in an abnormal vasculature that is often leaky and tortuous, with uneven distances between the vessels, blind ends and arteriovenous shunts [190]. Hypoxia occurs in regions where this disorganized vasculature is unable to transport sufficient amounts of oxygen via

red blood cells to meet the demands of the surrounding cancer cells. Depending on the underlying cause of the insufficient oxygen supply, tumor hypoxia may be divided into two main subgroups; chronic or acute hypoxia (reviewed in [191]). Chronic, or diffusion-limited, hypoxia is generally found in regions far from functioning blood vessels and typically last for hours or days. The most extreme cases of chronic hypoxia are associated with necrotic regions found in larger tumors, where the oxygen levels are so low they are close to anoxia. In contrast, acute hypoxia is typically caused by temporary occlusions in blood vessels or changes in red blood cell flux, and only last for minutes or a few hours. Acute hypoxia often occurs in cycles of repeated periods of hypoxia and subsequent reoxygenation, and is then referred to as intermittent or cycling hypoxia. Hypoxia induces resistance to conventional cancer therapies such as radiation therapy and some forms of chemotherapy [192], and may promote a more aggressive tumor phenotype through multiple mechanisms (**Figure 6**). Due to this, hypoxia generally correlates with a poor outcome for cancer patients.

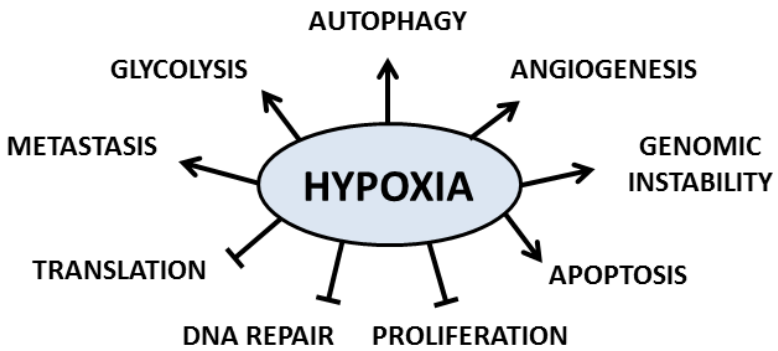


**Figure 6. Cellular responses to hypoxia.** Hypoxia promotes a more aggressive tumor type by stimulating processes involved in cancer progression and treatment resistance, and affects many aspects of cellular functions.

### Cellular adaptation to a hypoxic environment

As outlined in **Figure 6**, cellular adaptation to hypoxia is a complex response which affects many basic cellular processes [193], including metabolism, translation and transcription, in addition to cell cycle progression as described in the next section. Several of these responses are regulated by

the hypoxia-inducible transcription factors (HIFs), the unfolded protein response (UPR) and the mTOR (mammalian target of rapamycin) pathway.

HIFs are heterodimeric transcription factors composed of a constitutive β-subunit (ARNT) and an α-subunit (HIF1-, HIF2- or HIF3-α) (reviewed in [194]). HIF1 is the best studied of these and is conserved in all metazoans, and is the main activator of transcriptional responses to hypoxia. HIF1 activity is tightly regulated by proteasomal degradation of the α-subunit, mediated by its interaction with the von Hippel-Lindau complex (vHL). The interaction between vHL and HIF1 is dependent on the hydroxylation of HIF1α by oxygen-activated prolylhydroxylases (PHDs). When oxygen levels fall, the PHDs are inactivated, HIF1α is no longer hydroxylated and accumulates. This leads to activation of hundreds of downstream target genes of HIF1 including factors involved in tumor promoting pathways such as glycolysis, angiogenesis, metastasis, stem cell maintenance and immune evasion [195]. Among the best studied targets of HIF1 are *VEGF* (vascular endothelial growth factor), *CA9* (carbonic anhydrase 9) and *EPO* (erythropoietin).

While the HIF family regulates the transcription of genes involved in both tolerating and overcoming hypoxia, the UPR and mTOR pathways primarily modulate translational processes in response to hypoxic stress, though they also regulate other processes such as autophagy, apoptosis and metabolism [196-198]. Protein synthesis is an energy-consuming process, and inhibition of the overall rate of mRNA translation is thus an efficient method for energy conservation in hypoxic cells. Hypoxia-induced inhibition of the mTOR complex leads to decreased initiation of cap-dependent translation, while activation of the UPR by hypoxia results in decreased general translation initiation due to inhibition of GDP to GTP exchange in the translation initiation factor eIF2 after phosphorylation by the kinase PERK (protein kinase RNA-like ER kinase)[199]. Decreased translation may help the cells overcome hypoxic stress by conserving energy, but at the same time the cells are dependent on the efficient translation of essential genes, including several HIF targets that are required for hypoxia tolerance. Maintaining this balance is to a large extent regulated by the mTOR pathway, but there is also a high degree of crosstalk between HIF, mTOR and UPR signaling to promote hypoxia tolerance and cancer progression (reviewed in [196]).

### Cell cycle progression in hypoxic cells

In addition to preserving energy by limiting transcription and translation, cells also delay progression through the cell cycle in response to hypoxic stress. The cell cycle response to hypoxia is highly dependent on both the severity and duration of oxygen deprivation, and may also vary considerably between different cell lines. In anoxic conditions, cellular respiration is severely impaired [200], and all replication stops instantly [201]. In response to severe hypoxia, cells in late

S phase, G2 and M will continue through to G1 and arrest in late G1 phase [201, 202], though a hypoxia-induced G2 checkpoint arrest has been reported in HCT116 cells [203]. This arrest in late G1 is known as the oxygen-dependent checkpoint, and it is independent of HIF, p53 and Rb status [204-206]. While the molecular mechanism for induction of this checkpoint is not entirely determined [193], the CDK inhibitors p27 and p21 appear to play an important role in recovery from this checkpoint [205]. Cells may also accumulate in late G1 under conditions of moderate hypoxia, but in contrast to the oxygen-dependent checkpoint this arrest is dependent on HIF1α [207]. Furthermore, cells exposed to prolonged moderate hypoxia downregulate essential cell cycle regulators such as cyclin A[208, 209], D[210] and E[211] as well as Cdc25A[212, 213], and upregulate CDK inhibitors including p27[214] and p16[215]. This may play a role in limiting the progression from G1 phase, also by activating a pRb dependent arrest in mid-G1 [211, 216]. The late G1 arrest has also been shown to be dependent on GCN2 and PERK through regulation of p21 and HIF1α [217]. On the other hand, cells exposed to hypoxia in low-glucose conditions have recently been reported to traverse the G1 arrest and rather accumulate in S phase, a process linked to downregulation of HIF1α and p27 [218].

Following reoxygenation after exposure to severe hypoxia, cells arrested at the oxygen-dependent checkpoint at the G1/S border can be observed entering S phase in a synchronous wave, while the cells arrested in mid-G1 enter S phase with only slightly delayed kinetics as compared to normoxic cells[219]. In contrast to cells in G1 or G2, that tolerate hypoxia remarkably well, S phase cells struggle when exposed to severe levels of hypoxia, and are prone to hypoxia-induced cell death[201]. S phase progression tends to be slow or non-existent in extreme levels of hypoxia, with severely decreased replication initiation and elongation [220]. After prolonged exposure to near anoxic conditions cells lose their replicative potential and are unable to resume cell cycle progression following reoxygenation [221]. The effects on replication initiation and elongation are primarily linked to the inactivation of the enzyme ribonucleotide reductase (RNR). RNR is the enzyme that reduces ribonucleotides to their deoxyribonucleotide counterparts, thus providing the building blocks for both DNA replication and repair [222]. When oxygen levels fall below approximately 1000ppm, RNR activity drops as its enzymatic activity is dependent on free oxygen, resulting in replication stalling and activation of the DDR including cell cycle checkpoints [223, 224].

### Hypoxia, DNA repair and genomic instability

While much of the aggressiveness of hypoxic tumors is related to increased metastasis, angiogenesis and resistance to therapy, it is also due to hypoxic cells displaying a so-called “mutator” phenotype with a high degree of genomic instability [225]. This genomic instability was initially primarily attributed to elevated expression of fragile sites due to replication stress and DNA

damage induced by increased levels of ROS in connection to hypoxia-reoxygenation cycles [226], but more recently the profound impact hypoxia has on the function of several DNA repair pathways has also been implicated [227, 228]. The efficacy of MMR, NER and HR are all impaired in hypoxic cells due to decreased activity or levels of essential repair factors [227], and recent studies have suggested that also NHEJ function is reduced [229-231]. Among the HR factors found to be downregulated by prolonged hypoxia are Rad51, Rad52 and BRCA1 [230, 232-237]. Rad51 and BRCA1 expression is regulated by promoter binding of transcription factors in the E2F family, and under hypoxic conditions a shift from activating E2F1 to repressive E2F4 complexes takes place, resulting in decreased levels of transcription[234]. Hypoxia-induced downregulation of Rad52 expression appears to rather be regulated by microRNAs (miRs), in particular the hypoxia-regulated miR-210 [235]. In contrast, the downregulation of the MMR genes *MLH1* and *MSH2* in hypoxia has been connected to decreased levels of the transcription factor c-Myc [238], indicating that there are multiple transcriptional and translational mechanisms involved in the regulation of DNA repair pathways in response to hypoxia.

This impaired DNA repair function may sensitize hypoxic cells to exogenous DNA damaging agents as well as to the endogenous DNA damage induced by the stressful microenvironment surrounding them. For instance, reduced DNA repair capacity may explain much of the observed increase in radiation sensitivity of prolonged hypoxic cells compared to acute hypoxic cells[239]. While the oxygen effect still provides some protection for them compared to normoxic cells, these cells will remain repair deficient for several hours or even days after reoxygenation, making them even more sensitive to IR than normoxic cells. This decreased repair capacity thus provides yet another mechanism for explaining the success of fractionated radiation therapy [239], where cells that have been exposed to prolonged hypoxia may be reoxygenated before the subsequent dose is administered [240, 241]. The impaired HR function has also implicated the use of PARP inhibitors for selective targeting of hypoxic cells [220, 227], analogous to the increased sensitivity to these inhibitors in cells deficient in HR due to mutations in BRCA1/2 [242, 243]. However, in contrast to what is observed after prolonged hypoxia, where HR function is impaired, acute hypoxic cells have been reported to be more dependent on HR for survival following IR treatment than normoxic cells [244]. This may be linked to differences in the type of DNA damage induced by IR under hypoxia versus normoxia [245].

### Hypoxia and activation of the DDR

As mentioned previously, hypoxic cells struggle with progression through S phase, particularly when the level of hypoxia is severe. When oxygen levels are close to anoxia, progression through S phase is not only delayed but stops entirely, affecting both replication initiation and elongation,

and cell death is induced after just a few hours exposure. Under such conditions, a massive DDR primarily dependent on ATR/Chk1 mediated signaling is activated [224, 246], likely due to the formation of long stretches of ssDNA formed at stalled replication forks. This replication stress response is similar to the response to hydroxyurea or aphidicolin treatment with regard to activation of ATR/ATRIP, Rad17 and Nbs1, though it does not result in DSBs as measured by comet assays, 53BP1 foci nor formation of ATM foci [247]. Later studies found that ATM is activated by such severe levels of hypoxia, but displays a diffuse staining throughout the nucleus rather than form foci [248]. Accumulation of p53 was also observed in arrested S phase cells [224, 249], and has been implicated in induction of apoptosis in hypoxic regions (reviewed in [250]). While exposure to extreme hypoxia has not been found to induce any measurable DNA damage, reoxygenation after such treatment causes massive formation of DSBs with subsequent formation of ATM foci and phosphorylation of Chk2 [188]. Chk1 activation in hypoxic cells has been implicated in maintaining replication fork integrity and cell cycle arrest [251, 252], and consistent with this, cells display increased sensitivity to abrogation of Chk1 or ATR when exposed to severe hypoxia and subsequent reoxygenation [251, 253]. Chk1 inhibitors have therefore been suggested to selectively target hypoxic cells [220, 254], even though the role of Chk1 in moderate hypoxic conditions that do not induce replication stalling has not been elucidated, which led us to initiate the study in paper II. However, γH2AX has also been observed in response to moderate hypoxia (1% $O_2$) in epithelial cells, where it was found to be required for angiogenesis and cell proliferation [255]. Evidently, there is a strong connection between the DDR and adaptation to a hypoxic environment, though the interplay between these processes is still poorly understood.

## Aims of study

In order to achieve a better understanding of the biological functions of Chk1 and how they may be targeted to enhance cancer treatment, we initiated three closely related studies with the aim of determining the following:

- The role of p53 status for the effects of Chk1 inhibitors in combination with IR treatment in cancer cells (paper I).
- The effect of Chk1 inhibition in cells during and after prolonged or acute hypoxia, both in the presence and absence of IR treatment (paper II).
- How exposure to prolonged hypoxia may alter IR-induced G2 checkpoint signaling (paper III).

## Summary of papers

**Paper I**

**p53-dependent G1 arrest in 1st or 2nd cell cycle may protect human cancer cells from cell death after treatment with ionizing radiation and Chk1 inhibitors**

Inhibitors of Chk1 have been proposed to selectively sensitize p53-deficient cancer cells to DNA damage inducing agents by abrogating the G2 checkpoint, based on the hypothesis that these cells are more dependent on this checkpoint due to loss of a functional G1 checkpoint. While several previous studies have been conducted to determine whether this hypothesis holds true, these studies have mainly used chemotherapeutic agents rather than IR to induce DNA damage, few have used isogenic cell lines and results have varied, with most studies supporting the hypothesis, while others did not. To clarify this issue, we performed the study presented in paper I. In this study two isogenic cell systems (differing only in p53 status) were used, the osteosarcoma cell line U2OS-VP16 with Tetracycline-regulated expression of a dominant-negative version of p53 and the colon carcinoma cell lines HCT116$^{p53+/+}$ and HCT116$^{p53-/-}$. Chk1 function was abrogated by use of the inhibitors UCN-01 and Cep-3891, and the responses to combination treatments of Chk1 inhibitors and IR were assessed. HCT116$^{p53-/-}$ cells, but not HCT116$^{p53+/+}$, showed an increased sensitivity to IR in the presence of Chk1 inhibitors as measured by clonogenic survival assays, whereas U2OS-VP16 cells were radiosensitized by Chk1 inhibition independently of p53 status, with no additional effect observed in cells expressing dominant-negative p53. However, when measuring cell survival at three days after IR and exposure to Chk1 inhibitors, we observed a p53-dependent response in the U2OS cells, with increased cell death in cells expressing dominant negative p53. This p53-dependent response correlated with a p21-dependent G1 arrest in the second cell cycle after IR. However, the effects of p53 status on Chk1 inhibitor cytotoxicity within each isogenic cell system were minor compared to the difference in intrinsic sensitivity to Chk1 inhibitors between the cell lines, with HCT116 cells being very resistant and U2OS cells very sensitive. This would indicate that p53 status may not be the key predictor to Chk1 inhibition response.

**Paper II**

**The efficacy of CHK1-inhibitors is not altered by hypoxia, but is enhanced after reoxygenation**

Hypoxia is a hallmark of solid tumors, and correlates with a more aggressive disease with increased metastasis and resistance to therapy. In paper II, we investigated the potential of Chk1 inhibitors for selective targeting of hypoxic cells both as a monotherapy and in combination with IR. This study was based on previous publications showing that severe hypoxia activates a replication stress response involving activation of Chk1 and ATR [188, 251], and that cells irradiated under acute hypoxic conditions display an increased dependence on HR for survival [244]. During prolonged exposure to non-toxic levels of hypoxia, we found no enhanced cytotoxicity of Chk1 inhibitors despite activation of Chk1 and other DDR response proteins. In contrast, Chk1 downregulation by shRNA, which also kept Chk1 levels low during the initial 24 hours after reoxygenation, showed enhanced cytotoxicity. This indicated that cells may display increased dependence on Chk1 after reoxygenation. Consistent with this notion, administration of Chk1 inhibitors after reoxygenation following prolonged hypoxia resulted in enhanced γH2AX staining in S phase cells and decreased clonogenic survival. This increased sensitivity to Chk1 inhibition did not appear to be caused by enhanced unscheduled replication initiation, but rather seemed associated with more replication stalling as measured by EdU uptake and RPA phosphorylation. Interestingly, the increased sensitivity to Chk1 inhibitors was not only observed after exposure to hypoxia levels activating a DDR, but also after more moderate hypoxia that did not appear to cause replication stress. Inhibition of Chk1 radiosensitized hypoxic and normoxic cells to a similar extent, with a non-significant tendency for enhanced effect in hypoxic cells pointing to a slightly increased dependency on HR repair.

**Paper III**

**Impact of hypoxia on G2 checkpoint signaling**

Chronic hypoxia is known to impair several DNA repair pathways, including HR and MMR, which may be a major factor promoting genomic instability in tumors. The DNA damage induced G2 checkpoint is also a crucial factor in maintaining genomic stability, but how it is affected by prolonged hypoxia is not known. This led us to initiate the project presented in paper III. We found by microarray analysis that of a panel of 31 G2 checkpoint regulators, several were downregulated at the mRNA level by 24 hours exposure to moderate hypoxia, while others were unaltered or even slightly upregulated. On the protein level as measured by Western blotting, the general tendency was a downregulation of both positive and negative regulators of the checkpoint, in particular in response to more severe hypoxia. One notable exception was p21, which was upregulated by hypoxia. However, the prolonged exposure to hypoxia also resulted in an accumulation of cells in G1, where the expression of many of the G2 checkpoint regulators is at a minimum. To avoid the bias of cell cycle effects and accurately measure the protein levels in individual cells in each cell cycle phase, we used multiparameter flow cytometry. We found that the levels of cyclin B and Plk1 were decreased in G2 cells after exposure to prolonged hypoxia compared to the level in normoxic G2 cells, while cyclin A levels were unaltered. Consistent with this, the G2 checkpoint was stronger in cells irradiated after prolonged hypoxia than in normoxic cells, even though Chk1 signaling and response to inhibition of Chk1 appeared unaltered. In conclusion, altered expression of G2 checkpoint regulators by prolonged hypoxia may result in increased G2 checkpoint activation, and this may counteract rather than promote genomic instability.

# Discussion

## Experimental considerations

### Cell cultures and hypoxia treatment

The model system used throughout this thesis was cultured mammalian cell lines, primarily human cancer cell lines grown adherently in a monolayer. Cell cultures are one of the most frequently used model systems for studying biological responses with regard to cancer therapies, and some of the main advantages of this system includes the unlimited supply of homogenous material for DNA, RNA and/or protein analysis, the potential for long-term storage in liquid nitrogen and the ease of manipulation, whether by transfection, irradiation or addition of chemicals to the culture medium. However, these cultured cell lines have been passaged for years *ex vivo*, and have often lost several of their original characteristics in the process of adapting to proliferation in a monolayer under conditions of high oxygen (the oxygen level in normal room air and thus in humidified incubators is about 20-21%, in normal tissue generally 2-9%[256]) and diabetic glucose levels (glucose in DMEM medium is 25mM, 2h plasma glucose levels in a healthy person is <7.8mM according to the World Health Organization), in the absence of the hierarchical 3D structure and other cell types usually found in the tissue from which they were derived. Some of the observed discrepancies between preclinical and clinical studies may to a certain extent be explained by these "un-biological" conditions and selection for cells that thrive under them. It is therefore important to keep in mind that cultured cells may respond to various treatments in a different way than the tissues they were derived from. Even so, cultured cell lines are an invaluable tool in cancer research for understanding the underlying biological mechanisms in both cancer development and treatment.

Different cell lines may display very variable phenotypic and genetic characteristics, and respond differently to treatments such as hypoxia, Chk1 inhibition and irradiation, which is why we used several cell lines originating from various tissues and tumor types for our studies of these responses. Primarily the experiments in all three publications presented in this thesis were carried out with U2OS and HCT116 cells, but also HeLa, HT29 and BJ fibroblasts were used to ensure the general validity of the findings. U2OS is an osteosarcoma cell line originating from a moderately differentiated sarcoma of the tibia of a 15 year old girl, isolated in 1964[257]. It is a chromosomally highly altered cell line with chromosome counts in the hypertriploid range, and is wildtype for p53 and pRb[258, 259]. HCT116 is a near diploid cell line originally isolated as one of three subpopulations derived from a primary colonic carcinoma [260]. It is frequently used for knockout studies due to its suitability for targeted HR [261]. HeLa is an aneuploid cervical cancer cell line, and is also the oldest and one of the most well-studied cancer cell lines in use. HeLa cells contain the

human papilloma virus, leading to degradation of p53 and pRb through the activity of the viral E6 and E7 proteins, but otherwise the p53 and pRb pathways are intact [262]. HT29 is a cell line derived from a colon adenocarcinoma in 1964, with a hypertriploid chromosome number (www.atcc.org). BJ fibroblasts, a non-transformed human cell line derived from foreskin tissue (www.atcc.org), was also used for control experiments for publication I, as it in contrast to the cancer cell lines does not have any known mutations in pathways involved in the DDR. All the cell lines were mycoplasma-tested regularly and genotyped by STR technology to ensure their correct identity.

In publication II and III, the cell cultures were exposed to various levels of hypoxia (0.2% or ~0.03% $O_2$) for durations up to 24 hours, for which an InVivo$_2$ 200 chamber (Ruskinn) was used. This chamber functions like an airtight cell incubator, with regulated temperature and humidity, and also allows for handling of the cell cultures with minimal disturbance of the environment. The gas was regulated by a mixer with sensors for $O_2$ and $CO_2$ levels, which ensured flushing of the chamber with $N_2$ and $CO_2$ when needed to maintain the set level of these gases. To achieve the lowest level of hypoxia (~0.03% $O_2$) a mixture of $H_2/N_2$ gas was used in combination with the installed Palladium catalyst for removal of the remaining traces of free $O_2$. The gas mixer for the hypoxia chamber measures the oxygen level in the gas phase at one minute intervals, but the oxygen level in the medium of the cells was not determined at any time during our experiments. This would have required the use of oxygen probes inside the chamber, for which the necessary tubing and connections were not available in our chamber and which if connected could have interfered with the stability of the hypoxic environment. For our purposes, we found it adequate to depend on the chamber measurements and settings, and rather analyze more in detail the biological responses of each of the cell lines with regard to survival and effects on cell cycle progression and potential activation of a DDR. In addition, we tried to minimize variability between experiments by plating out cells at the same density and in the same volumes, as these factors may influence on oxygen availability and consumption, and thus the severity of the hypoxia [263]. In contrast to glass dishes, which are typically used for experiments where the exact oxygen level is of major importance and/or one desires to reach near anoxic conditions (less than 100 ppm $O_2$), plastic dishes bind oxygen and releases it gradually, resulting in delayed kinetics of achieving an equilibrium between the hypoxic gas phase and the cell culture medium [264]. Within the timeframe of our experiments (up to 24 hours hypoxic exposure) we did not observe decreased cell survival or complete cell cycle arrest, indicating that the oxygen level in the cell culture medium remained above 100 ppm throughout the hypoxic exposure [265], even with the use of $N_2/H_2$ gas and a Palladium catalyst to achieve near anoxic conditions. However, with longer exposures the

available oxygen from the plastic dishes would be consumed, and this would probably result in decreased cell survival and complete replication arrest, consistent with what has previously been reported for cells treated with near anoxic levels of hypoxia [188, 201, 219, 224, 265], though we did not attempt to test this out as the gas consumption for the chamber was very high at the most severe hypoxia setting (~0.03% $O_2$).

To avoid reoxygenation of the cells, all lysates and flow cytometry samples were harvested inside the hypoxia chamber with solutions that had been incubated in containers with open lids in the chamber throughout the experiment. Even so, the final step in some of the assays had to be done in normoxia, such as the fixation of the flow samples with ice-cold ethanol after centrifugation, but this was a process of less than two minutes per sample. For lysates analyzed by Western blotting, we could not detect any difference in the results for samples harvested in hypoxia or within the initial few minutes after reoxygenation, even for the extremely labile HIF1α, though at fifteen minutes after reoxygenation this protein was non-detectable. For more stable proteins, such as Chk1, Plk1, cyclin B and cyclin A, we did not observe any change in protein levels at such times after reoxygenation, though there was a gradual increase observed at 3-6 hours (paper III). Based on these observations, for the checkpoint activation studies in paper III we allowed the cells to be reoxygenated 15 minutes before irradiation rather than irradiate them immediately after removal from the hypoxia chamber, as we considered this short exposure to normoxia unlikely to have a major impact on the G2 checkpoint regulation. In support of this, we also observed a stronger G2 checkpoint in cells irradiated during prolonged hypoxia (20 hours of 0.2% $O_2$) compared to cells irradiated during acute hypoxia (3 hours of 0.2% $O_2$) (unpublished results), though these cells displayed a weaker G2 checkpoint compared to normoxic cells due to less DNA damage induced by the radiation treatment because of the oxygen effect.

### Abrogation of Chk1 function

The development of small molecule inhibitors of Chk1 has become a priority for several pharmaceutical companies due to their potential clinical applicability, and many patents for such compounds have been applied for [266, 267]. These compounds have various chemical properties and selectivity for Chk1, but most of them are ATP competitive structures that bind the active site [266, 267]. They therefore often inhibit other kinases in addition to Chk1, but with different selectivity, which is why it is so important to test multiple Chk1 inhibitors to ensure that the observed effects are truly dependent on Chk1 and not the other kinases targeted by the inhibitor. In publication I we used the Chk1 inhibitors UCN-01 and Cep-3891, in study II UCN-01 and AZD7762. UCN-01 (7-hydroxystaurosporine) was the first Chk1 inhibitor to be discovered[268], and also the first to reach clinical trials [269]. Though primarily used as a Chk1 inhibitor [270], it was

originally isolated as a PKC (protein kinase C) inhibitor [271, 272] and additionally inhibits PDK1 (pyruvate dehydrogenase kinase 1) and AMPK (AMP-activated protein kinase) [273]. Cep-3891 efficiently abrogates Chk1 function ($IC_{50}$ 4nM), but also targets TrkA ($IC_{50}$ 9nM), MLK1 ($IC_{50}$ 42nM) and VEGFR2 ($IC_{50}$ 164nM) *in vitro* [63]. Unlike UCN-01 and AZD7762, it has never been tested in clinical trials due to poor stability *in vivo*, but has been shown to abrogate S and G2 checkpoints after IR [274] in addition to causing increased replication initiation and DNA breakage in unperturbed cells [121, 154]. AZD7762 is a newer and more specific Chk1 inhibitor, but in addition to Chk1 it also targets Chk2 to a similar extent ($IC_{50}$ 5nM and <10 nM, respectively)[275].

While inhibitors are a quick and efficient way of abrogating Chk1 function, they are also unspecific, and in some cases result in different responses than those observed with knockdown or knockout studies, where the protein is absent rather than inactivated. Knockdown of the protein of interest is achieved by the use of interfering RNA (RNAi), either in the form of vector-based short hairpin RNA (shRNA) or chemically synthesized small interfering RNA (siRNA). While we used transient transfection of siRNA for knockdown of p21 in paper I, and often have used siRNA for knockdown of other proteins including Chk1 for many experiments previously with good results [73, 121], a U2OS cell line with Tetracycline-inducible expression of Chk1-shRNA was made for the studies in paper II. Chk1 knockdown is in itself quite toxic to the cells, so uneven transfection efficiency between single cells in a population and between experiments would result in an unfortunate selection for cells where Chk1 knockdown was inefficient in clonogenic survival assays. In order to minimize such variability, single clones of stably transfected cells were tested for efficient knockdown of Chk1 in response to Tetracycline addition. Out of an initial 24 clones, only two were further tested for radiation sensitivity and response to hypoxia based on their normal background levels of Chk1 in the absence of Tetracycline and efficient knockdown of Chk1 in the presence of this drug (Western blotting showed levels of Chk1 to be less than 1/16 of background levels following 48 hours of Tetracycline treatment). One of these clones was discarded from further experiments due to increased radiation sensitivity also in the absence of Tetracycline when compared to parental U2OS, while the B1-clone used for the experiments in paper II did not deviate from the parental cell line in any way tested (clonogenic survival responses to both radiation and hypoxia, flow cytometry measurements of cell cycle progression and protein levels assessed by Western blotting). In addition to the obvious advantages of having a clonal population of cells with drug-inducible expression of shRNA, shRNA is generally also more efficient than siRNA and may result in less off-target effects [276].

Studies of the immediate responses to loss of Chk1, such as the initial burst of replication initiation and mitotic entry, require the use of inhibitors of this kinase, as knockdown with RNAi takes several

hours or even days to achieve sufficiently low levels of Chk1 protein. However, RNAi is generally more specific, even though this method also may result in off-target effects. Ideally, both techniques should be applied when possible, as we have done in publication I and II.

### Cell death and survival assays

In radiation biology, clonogenic survival assays have generally been considered the "gold standard" for determination of treatment efficacy, only surpassed by *in vivo* mouse models [108]. This assay measures the fraction of cells that are able to propagate and form new colonies through several rounds of cell division, which is thought to best mimic the fraction of cells that would continue to proliferate indefinitely in a tumor, and thus remain a threat to the patient. However, treatment efficacy may also be measured by assaying the fraction of dead and viable cells at a shorter time-point following exposure to cytotoxic agents, typically 2-3 days, such as with the Sytox green viability assay used in publication I, or with assays measuring apoptosis, such as the TUNEL assay. Viability and apoptosis assays are generally quicker and less laborious than the clonogenic survival assays to perform, and though there are exceptions, in particular for the apoptotic assays [108], they may correlate with observed responses *in vivo* [277, 278]. However, apoptosis assays are primarily only applicable for cell lines that are particularly prone to this mode of cell death, such as hematopoietic cells, and were therefore not suitable for our experiments. In publication I, both the clonogenic survival assay and the Sytox green (a non-permeable nucleic acid stain that only stains cells with a ruptured membrane) viability assay were used to assess the efficacy and p53-dependence of Chk1 inhibition in combination with IR in U2OS cells, with only the cell viability assay showing a p53-dependent effect. Such discrepancies between clonogenic survival assays and short-term viability assays have been observed with regard to p53 status and Chk1 inhibition previously [279], and may be one of the reasons for some of the contradictory findings published on the subject. Since p53 regulates both cell cycle arrest and apoptosis, lack of this tumor suppressor may both promote as well as protect from cell death after DNA damage, and as a consequence the mechanism and timing of cell death may vary, but the fraction of cells that are able to form new colonies does not necessarily do so. The observed second cycle G1 checkpoint in p53-competent U2OS cells is likely protecting irradiated cells from death by necrosis or mitotic catastrophe, as measured by the viability assay three days after irradiation, but may also be the first step towards DNA-damage induced senescence or activation of p53-dependent apoptosis. Even so, the mode and kinetics of tumor cell death may be important for the systemic response to treatment with regard to inflammation, fibrosis and other late effects, and should be taken into account when considering the validity of cell viability and clonogenic survival assays for prediction of *in vivo* responses.

### Cell cycle progression measurements

Treatments with hypoxia, Chk1 inhibition and IR all have in common that they affect cell cycle progression. To measure the cell cycle distribution and progression, we used multiparameter flow cytometry, a technique that allows for the assessment of DNA content and protein modifications/levels within individual cells in a large population (typically 10 000 to 100 000 cells are measured for each sample) within a few minutes. Flow cytometry can also be used to analyze subpopulations within a large cell culture sample by gating for the populations of interest, such as mitotic cells or γH2AX positive S phase cells. The use of this technique for protein measurements is discussed in the next section. Fluorescent dyes staining DNA were used for determining the fraction of cells with 2n (G1 phase), 4n (G2 or M phase) or intermediate DNA content (S phase cells). The DNA stains used were primarily Hoechst 33258 and to a lesser extent Cell Cycle 633, depending on which other fluorescent dyes they were to be combined with. With the flow cytometer we used for our experiments, Hoechst 33258 was preferred as it generally gave DNA histograms with narrower peaks and better separation of the distinct cell cycle phases than the Cell Cycle 633 stain, but only the latter fluorescent dye could be combined with the EdU staining or the barcoding technique (see below). To separate G2 cells and mitotic cells an antibody against the phosphorylated Serine 10 residue of histone H3 was used as a mitotic marker [280]. The microtubule inhibitor Nocodazole was used to arrest cells in mitosis for up to 24 hours to measure the accumulation of cells that had avoided or overcome the G2 checkpoint arrest and also to study G1 arrest, both in response to IR and hypoxia. Longer durations of Nocodazole treatment were found to result in a marked increase in the sub-G1 fraction, and could therefore not be used to assess G2 checkpoint recovery at such late times after IR. While the fraction of cells in S phase could be calculated to some extent by the DNA content, this method does not separate between actively replicating and arrested S phase cells, so for that purpose the nucleotide analog EdU was added to the medium of the cells. For determining the fraction of actively replicating cells, a short (10 minute) pulse of EdU was given, while a one hour pulse of a lower concentration of EdU was used for the more accurate quantification of replication rate in the cells (paper II), as this gave more consistent values.

### Measurements of protein levels and modifications

Three different techniques were used in this thesis to study the levels and post-translational modifications of proteins: Multiparameter flow cytometry, Western blotting and immunofluorescence imaging (IF). While all three methods rely on the use of specific antibodies, they each have distinct properties which make them useful for different purposes. Most antibodies work well for Western blotting, with the exception of those that only bind the protein in its native

conformation, and the gel electrophoresis process separating the proteins by size minimize the problem of unspecific binding to other proteins than the one of interest. However, Western blotting is not so suited for measurements of protein levels or modifications that only apply to a small fraction of the total cell population. This method will not be able to discriminate between large variations in a small fraction of the total population and a small variation in the whole population, as the observed band is an indication of the mean protein level for a large population of cells. Multiparameter flow cytometry is much better suited for studies with large variations within the studied cell population, as it measures the signal within single cells, though there are issues with the limited number of antibodies available that are compatible with this technique. So far we have therefore only tested with flow cytometry a few of the G2 checkpoint regulators found to be downregulated by prolonged hypoxia according to the Western blotting and microarray data in paper III. In particular the results for cyclin A and cyclin B in this study show the importance of taking cell cycle effects into account, as only the latter was found to be downregulated in G2 phase in the flow cytometry data, while both appear to be downregulated when assessed by Western blotting using whole cell lysates. This issue may be overcome by synchronizing the cells with methods such as the mitotic shake-off technique, though this is not easily achieved in studies such as ours where cells are treated with prolonged hypoxia, as such cells tend to accumulate in G1 and not enter S phase (paper II and III). One could in principle sort cells by FACS (fluorescence-activated cell sorting) according to cell cycle phase and then lyse the fractions for Western blotting analysis, but this would require very large sample sizes and is far more laborious than the direct analysis with flow cytometry. Flow cytometry thus remains an invaluable tool for acquiring data on protein levels and modifications within sub-populations of cells. However, this technique is not suited for analysis of subcellular localization or foci formation, for which IF has to be used. In general, the antibodies that work well with IF also work for flow cytometry, and the two techniques may be combined by sorting the cell population of interest onto microscope slides using FACS followed by microscopic analysis [281].

### Measurement of hypoxia-mediated changes in mRNA expression

For the study of mRNA changes in response to prolonged hypoxia, Illumina microarrays covering the whole human genome were used. The results acquired with this method may not be as reproducible as those one could achieve using real-time PCR (RT-PCR) or Northern blotting with multiple probes for each of the genes of interest, as was evident by the variability in the expression pattern found for each gene in those cases where multiple probes were available (results not shown). For our purpose the whole pattern of variability for the G2 checkpoint regulators was a more important issue than the exact regulation on the transcriptional level for each gene, and for

this we found the microarray data to be a useful indicator, while we prioritized the quantitation of the protein levels of these genes with Western blotting and flow cytometry. The microarray data in paper III were originally produced for a different study using the cervical cancer cell lines HeLa, SiHa and CaSki [282], while the U2OS cell line was added to the setup for our study. While there was some variability in the results obtained with these different cell lines, most of the genes were found to be differentially expressed in a similar pattern in response to hypoxia, and as expected many of the most upregulated genes were known hypoxia-regulated genes such as *CA9* and *VEGF*.

## General discussion

### p53 status and sensitivity to Chk1 inhibition

The initial rationale for using Chk1 inhibition for selective sensitization of cancer cells to DNA damaging agents was based on the theory that cells that lack a functional G1 checkpoint would depend more on the S and G2 checkpoints for survival, and abrogation of these latter checkpoints would thus selectively target p53-mutant cells. This also remains the rationale for targeting of triple negative breast cancer by Chk1 inhibition, as these tumors display a high rate of p53 mutations[283], though other factors such as oncogene-induced replication stress [185, 187] are emerging as other potential genetic markers for increased efficacy of such inhibitors.

Our study in paper I indicated that there is indeed an enhanced effect of Chk1 inhibition in cells lacking p53 compared to their p53 wildtype counterparts, though this study also showed that the cytotoxic effects of Chk1 inhibition alone were much greater in the U2OS than the HCT116 cell line, irrespective of p53 status. Like all cancer cells, these cell lines harbor various mutations that have accumulated through their process towards malignant transformation, and in particular for U2OS it is evident that the p53 pathway is not entirely functional. While this cell line does activate expression of p21 in response to IR, the response is much slower than what we observed in HCT116 cells, and this was also evident in the microarray data in paper III, where transcription of *CDKN1A* (p21) was strongly upregulated in HeLa, but not in U2OS after 24 hours treatment with 0.2% $O_2$. This delayed expression of p21 is likely the reason for the failure to arrest in G1 in the first cell cycle after IR, while the cells have managed to accumulate sufficient p21 in the second cell cycle to arrest there, as observed in paper I. It was also interesting to notice the difference between HCT116 and U2OS with regard to the radiosensitizing effect of Chk1 inhibition. HCT116$^{p53-/-}$ but not HCT116$^{p53+/+}$ cells were affected, whereas U2OS cells displayed increased radiation sensitivity regardless of p53 status, consistent with an aberrant regulation of this pathway. This may be caused by mutations in genes encoding proteins involved in the regulation of p53 such as Mdm2.

Indeed, U2OS has been found to overexpress Mdm2, but this study found HCT116 to do the same [259], and in contrast to U2OS, the HCT116 cell line is able to activate a G1 checkpoint immediately after IR (paper I).

Compared to other cell lines, U2OS cells seem sensitive to inhibition of Chk1, whereas HCT116 cells are exceptionally resistant (paper I, paper II and unpublished observations). The resistance of HCT116 to the detrimental effects of CHK1 inhibition may be coupled to the mutation in *MRE11* found in this cell line [284]. DNA DSB formation following inhibition of Chk1 has been shown to be dependent on the activity of the endonuclease Mus81/Eme1 [285], and Mre11 has been shown to be involved in degradation of stalled replication forks [286-288], so it could be that the decreased Mre11 function in HCT116 cells prevents excessive DNA resection and DSB formation following Chk1 inhibition. Indeed, a recent study showed that Mre11 is critical for the sensitivity of cells to Chk1 inhibition [289]. Another factor linked to increased sensitivity to inhibition of Chk1 is high levels of replication stress and genomic instability as measured by γH2AX staining in S phase during unperturbed cell cycle progression [186], though the correlation was rather inconsistent for the various cell lines tested in this study. For U2OS and HCT116 cells we observed far lower levels of S phase γH2AX in the prior than the latter cell line (paper II), indicating that in the case of these cell lines the correlation is inverse. High levels of cyclin B1 [290] and deficiency in the FA repair pathway [291] have also been implicated in promoting sensitivity to Chk1 depletion, though neither of these factors appear to be able to explain the differences between HCT116 and U2OS cells. What potentially may be a major factor is that U2OS cells have been shown to undergo aberrant mitosis and fail to activate the spindle checkpoint in response to Chk1 inhibition [292], making them prone to death by mitotic catastrophe, especially when Chk1 inhibition is combined with loss of p53. However, as a tetraploid cell line it may also be susceptible to p53- and p38 MAPK-dependent cell death after Chk1 depletion [293, 294]. These findings are consistent with our observations in paper I that the kinetics and mechanisms of cell death may be dependent on p53 status in U2OS cells.

### Hypoxia and sensitivity to Chk1 inhibition

One of the more surprising findings in paper II was the observation that cells were more dependent on Chk1 after reoxygenation than during hypoxia, even though it was only during the severe hypoxia treatment that the cells activated a replication stress response involving Chk1. In addition, the increased sensitivity was also observed following prolonged moderate hypoxia (0.2% $O_2$), where there was no activation of any DDR components nor signs of replication stress. Consistent with this, a recent publication showed reoxygenated cells to be particularly sensitive to inhibition of ATR also after moderate hypoxia [295], not only after severe hypoxia as had been reported

previously [224, 251]. Furthermore, the cells displayed increased sensitivity as measured by the fraction of γH2AX positive cells at 24 hours also if the Chk1 inhibitors were added at 6 hours after reoxygenation (paper II and results not shown), which is long after replication has resumed in the reoxygenated cells, indicating that this effect is distinct from the previously reported role of Chk1 in reoxygenation-induced replication restart after periods of severe hypoxia [253]. For the clonogenic survival assays showing increased sensitivity to Chk1 inhibition after reoxygenation, Chk1 inhibitors were also not administered immediately after reoxygenation, but up to 45 minutes later, as the cells had to be plated out after removal from the hypoxia chamber. Together, these results indicate that the increased dependence of Chk1 after reoxygenation is due to lack of one or more factors that are downregulated or inactivated by prolonged hypoxia and which require several hours to regain their level or activity, likely involving transcriptional or translational mechanisms. Among the factors reported to be downregulated by prolonged hypoxia are the MCM proteins, with subsequent reduced loading of replication complexes [221, 296]. Since decreased loading of MCM complexes has been implicated in promoting sensitivity to replication stress [151], this may be a factor promoting increased sensitivity to Chk1 inhibitors, yet we did not observe decreased loading of MCM complexes in our experiments (paper II). None the less, there may be other factors involved in replication that are decreased to such an extent by prolonged hypoxia that replication fork stalling is increased, as we observed to be the case for reoxygenated cells exposed to Chk1 inhibitors (paper II). Furthermore, prolonged hypoxia is known to impair several DNA repair pathways (reviewed in [225, 228, 297, 298]), and this decreased DNA repair capacity may last for hours or even days after reoxygenation, depending on the duration and severity of the hypoxia treatment. Since inhibition of Chk1 induces replication stress, it could be that the decreased repair capacity of reoxygenated cells is the cause of the elevated sensitivity to Chk1 inhibition, rather than the replication stress induced by hypoxia (see next section).

While transcriptional and translational responses regulating DNA repair pathways and replication factors may explain why reoxygenated cells are particularly sensitive to inhibition of Chk1, they do not explain why hypoxic cells are not so. In paper III, we observed that prolonged hypoxia led to downregulation of several cell cycle regulators and DDR proteins, consistent with previous reports (reviewed in [193, 298]). Since the detrimental effects of Chk1 inhibition are primarily due to elevated CDK activity mediated by accumulation of Cdc25A [139], the hypoxia-induced downregulation of Cdc25A and several cyclins (see introduction and paper III) in combination with the accumulation of cells in G1 phase and upregulation of p21 and p27 (paper III and [205]) probably limits the deleterious effects of Chk1 inhibition during hypoxia. Even so, decreased CDK activity in hypoxic cells is likely not the only explanation, as at least cyclin B levels were decreased

for several hours after reoxygenation (paper III), while increased phosphorylation of H2AX in response to Chk1 inhibition was observed within 90 minutes in reoxygenated versus normoxic cells (paper II). Interestingly, the downregulation of Cdc25A and suppression of replication initiation in hypoxic cells was recently reported to be dependent on ATR activity [299]. On the other hand, previous studies have implicated miRs and p21 in this process [212, 213], while depletion of ATR has been shown to not affect replication arrest in extreme hypoxia, but rather be involved in replication restart after such stress [251]. Some of these discrepancies may be due to differences in the hypoxia treatments used, as at least the latter study was conducted with oxygen levels so low that both initiation and elongation of replication was inhibited due to lack of deoxynucleotides caused by inactivation of RNR [221, 251], whereas only replication initiation but not elongation was affected by the hypoxia in the study by Martin *et al.* [299]. Consistent with the findings in this latter study, our results also point to the activation of DDR signaling involving ATR and Chk1 in hypoxic conditions that do not induce either complete cell cycle arrest or cell death. Rather, the activation of this DDR appears to be reversible, and the γH2AX observed in these cells is most likely a marker for activated ATR at RPA-coated ssDNA at stalled replication forks, not for DSBs. In the absence of other sources of replication stress these cells will likely be able to resume replication following reoxygenation, provided the duration of the hypoxia is not so long that replication forks collapse. In this regard, the hypoxia-induced downregulation of essential factors involved in HR and maintenance of fork stability, including Chk1 itself, may be one of the reasons for such fork collapse occurring. Interestingly, ATR-dependent phosphorylation of H2AX has been shown to be induced in response to moderate hypoxia and be required for regulation of angiogenesis [255], while other recent studies have shown ATR, ATM and DNA-PK to be involved in the regulation of HIF pathways [295, 300, 301]. These studies and our own indicate that there is an extensive interplay between the DDR and the cellular response to hypoxia, though there are still many unanswered questions remaining on this subject.

### Hypoxia, G2 checkpoint signaling and genomic instability

Considering the effect hypoxia has on DNA repair pathways, cell cycle regulators and activation of DDR, it is not surprising that hypoxia promotes genomic instability (reviewed in [225]). In addition, hypoxia is known to promote p53-dependent apoptosis (reviewed in [250]), resulting in selection of cells that lack this tumor suppressor and thus the DNA damage-induced G1 checkpoint, making them particularly dependent on the G2 checkpoint for maintenance of genomic integrity as we addressed in paper I. The stringency of the G2 checkpoint depends on the balance between negative and positive regulators, and both the levels and activities of many of these are altered by prolonged exposure to hypoxia (paper III and references therein). In paper III, we found that while

several of the factors promoting G2 checkpoint activation are downregulated by prolonged hypoxia, this was also the case for many of the factors that promote mitotic entry, including cyclin B and Plk1. Prolonged hypoxia may thus likely alter the balance between negative and positive regulators of the G2 checkpoint, and consequently the stringency of this checkpoint. Following 20 hours incubation at ~0.03% $O_2$ we observed increased G2 checkpoint activation in U2OS cells. Although it remains to be tested whether similar effects are found in other cell lines and after different hypoxic conditions, these results suggest that prolonged hypoxia causes a more stringent G2 checkpoint that counteracts rather than contributes to hypoxia-induced genomic instability.

The exact role of or mechanism for hypoxia-induced downregulation of these cell cycle regulators is not clear, though Plk1 has been implicated in regulation of the mTOR pathway [302], while expression of cyclin B1 has been reported to be regulated by eIF4E and is decreased in response to mTOR inhibition [303]. Decreased levels of cyclin B are also observed in freeze tolerant wood frogs in response to anoxia [304, 305] and is there thought to promote stress tolerance by decreasing cell cycle progression. In general, hypoxia mediates cell cycle delay by upregulation of CDK inhibitors and downregulation of cyclins, and at least cyclin D1 is known to be a HIF target [210], though HIF-independent mechanisms have also been shown to be involved [306]. Based on these studies and our own findings, it is not unlikely that the balance of positive versus negative regulators of mitotic entry favor a more stringent G2 checkpoint in hypoxic cells. Even so, the decreased repair capacity of cells exposed to prolonged hypoxia may also contribute to delayed mitotic entry of irradiated cells. While our findings indicate a more stringent G2 checkpoint in hypoxic cells mediated by decreased levels of Plk1 and cyclin B in G2 cells, we have not yet assessed the stringency of the G2 checkpoint as measured by number of γH2AX foci remaining as cells enter mitosis, as was done in a recent study from our research group [281]. However, such studies are under way, and will help to determine whether hypoxic cells merely abrogate the G2 checkpoint with delayed kinetics due to impaired repair capacity or if they enter mitosis with less damage than normoxic cells after irradiation.

## Concluding remarks

As mentioned in the introduction, cancer is a disease that is both caused by and treated with DNA damage. Understanding the molecular basis of cancer therefore requires the understanding of the cellular responses to DNA damage, and this knowledge may help us find new potential targets for improvement of cancer therapy. Most importantly, we need to find those aspects of the DDR that differ between cancer cells and normal tissue to optimize the therapeutic ratio, thus improving chance of survival and life quality for the patients.

In this thesis, two main tumor specific traits and the potential for targeting them by inhibition of Chk1 were addressed, namely p53 status and hypoxia. In paper I, we found p53 status to be important for radiosensitization by Chk1 inhibition, as previously suggested, though this study also showed p53 status to be of lesser importance in mediating response to Chk1 inhibition than other traits the cancer cells had acquired during their process towards malignant transformation. In paper II, we addressed the potential for targeting hypoxic tumor cells by inhibition of Chk1, and found that while enhanced sensitivity to inhibition of Chk1 is not observed in hypoxic cells, reoxygenated cells do depend more on this kinase for survival. In addition, this study also showed that hypoxic and normoxic cells are radiosensitized to a similar extent by Chk1 inhibition. In paper III, we studied the effect of prolonged hypoxia on the Chk1 dependent G2 checkpoint, based on observations made during the work on paper II that hypoxia alters the levels of several G2 checkpoint regulators. We found that activation of the IR-induced G2 checkpoint may be enhanced by prolonged hypoxia, which may counteract rather than promote hypoxia-induced genomic instability.

These studies have highlighted some of the aspects regarding the DDR and the cellular responses to hypoxia, and the extensive crosstalk existing between these pathways. In order to exploit the full potential of Chk1 inhibitors in the clinic, we need to learn more about these processes, both *in vitro* and *in vivo*, so that we can determine which patients and what type of tumors will respond best to such treatments, and avoid giving such agents to non-responders. Detrimental side effects and poor pharmacokinetics have been an issue in previous clinical trials with Chk1 inhibitors [174, 180], and currently there are only two Chk1 inhibitors in ongoing trials (www.clinicaltrials.gov). Hopefully, these and future trials will yield more promising results for the use of Chk1 inhibitors in the clinic.

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